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RESEARCH ARTICLE

Immobilization of lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* by adsorption on variously grafted silica gels

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Abstract

Twenty variously surface-modified mesoporous silica gels were studied as carriers for immobilization by hydrophobic adsorption of the lipases from Rhizomucor miehei *(*Rm*L) and* Thermomyces lanuginosus *(*Tl*L). Several of the surface-modified silica gels studied proved to be advantageous supports for* Rm*L and* Tl*L resulting in novel biocatalysts of high activity and enantioselectivity in the kinetic resolution of racemic 1-phenylethanol rac-*1*. The fact that it were different grafting methods which led to the most efficient supports for* Rm*L and* Tl*L indicated that the selection of optimal support for the immobilization of a particular lipase cannot be predicted.*

Keywords

silica gel · surface modification · kinetic resolution · lipase · immobilization · Rhizomucor miehei · Thermomyces lanuginosus

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1 Introduction

Biocatalysis with isolated enzymes can simplify and improve organic syntheses even on an industrial scale [1]. Lipases (EC 3.1.1.3) are essential in the digestion, transport and processing of lipids (e.g. triglycerides, fats, oils) in most, if not all, living organisms. Lipases are versatile biocatalysts which can provide regio- and enantioselectivity in a wide range of reactions [2, 3]. Consequently, they are one of the most extensively utilized biocatalysts in organic synthesis [4, 5].

Immobilization can enhance activity, thermal and operational stability, and also reusability of enzymes which are essential advantages in industrial applications [6, 7]. Among the many available immobilization methods, including adsorption, covalent attachment to solid supports and entrapment within polymers [8–11], hydrophobic adsorption onto suitable carriers was found to be an efficient way not only for immobilization but also for the separation of lipases [12]. Lipases immobilized by various procedures proved to be useful both in batch mode and in continuous-flow biotransformations [13–16].

Since we have found that surface-modified silica gels proved to be efficient supports for adsorptive immobilization of lipases A and B from *Candida antarctica* (*Ca*LA and *Ca*LB) and those from *Pseudozyma aphidis* (*Pa*L) [12], *Pseudomonas fluorescens* (Lipase AK) and *Burkholderia cepacia* (Lipase PS) [17], we were prompted to extend our studies to lipases from the thermophilic filamentous fungi *Rhizomucor miehei* (*Rm*L) and *Thermomyces lanuginosus* (*Tl*L) supported by variously grafted silica gels as well.

2 Experimental section

2.1 Chemicals and enzymes

Racemic 1-phenylethanol *rac*-**1** and vinyl acetate were obtained from Sigma-Aldrich. All solvents of analytical grade or higher were products of Merck. Solutions of lipases from *Rhizomucor miehei* (>20000 U g^1, Cat. no: L4277) and *Thermomyces lanuginosus* (>100000 U g^1, Cat. no: L0777) were purchased from Sigma-Aldrich. Davisil® 250 [40-63 μm] was the product of W. R. Grace & Co. Etched silica gel was prepared from Davisil® 250 by shaking in ethanol containing cc.

NH_4OH (0.5 v/v%) for 4 days. Surface functionalized silica gels were the products of SynBiocat Ltd.

2.2 Analytical methods

GC analyses were carried out on Agilent 4890 instrument equipped with FID detector and Hydrodex β-6TBDM column (25 m × 0.25 mm × 0.25 μm film with heptakis-(2,3-di-O-methyl-6-O-*t*-butyldimethylsilyl)-β-cyclodextrine; Macherey&Nagel) using H_2 as carrier gas (injector: 250˚C, FID detector: 250˚C, head pressure: 12 psi, 50:1 split ratio, oven: 120˚C, 8 min).
GC: t_r (min) for *rac*-**1** and *rac*-**2**: 4.0 [(*S*)-**2**], 4.4 [(*R*)-**2**], 5.8 [(*R*)-**1**], 6.0 [(*R*)-**1**].

2.3 Adsorption of enzymes on surface modified silica gels

Lipase solution (*Rm*L or *Tl*L; 1.25 mL) was dissolved in Tris buffer (11.25 mL, 100 mM, pH=7.5, ionic strength controlled with NaCl) then surface functionalized silica gel (250 mg, as indicated in Tab. 1 and Tab. 2) was added to the solution. The resulting suspension was shaken at 400 rpm and 4˚C for 18 h. The supported lipase was filtered off with a glass filter (G4), washed with 2-propanol (5 mL, twice), hexane (5 mL), dried at room temperature (2 h) and stored at 4˚C.

2.4 Enantiomer selective acetylation of racemic 1-phenylethanol *rac*-1 in shaked vials

To a solution of the racemic 1-phenylethanol *rac*-**1** (101 mg; 0.828 mmol) in hexane/*t*-butyl methyl ether/vinyl acetate 6/3/1 (2 mL) immobilized lipase (50 mg) was added in a sealed amber glass vial and the resulting mixture was shaken (1000 rpm) at 30˚C for 4 hours. The reactions were analyzed by GC and TLC after 1, 2, 4, and 24 hours.

3 Results

Modification of the surface of a mesoporous silica gel (Davisil® 250; particle size: 40-63 μm, pore diameter: 25 nm) with a selection of mono- and disubstituted alkoxysilanes as reported earlier [12] enabled us to prepare nineteen mechanically stable silica based supports of varying hydrophobicity.

Although lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* are commercially available in immobilized forms [e.g. the Cross-Linked Enzyme Aggregate (CLEA) or Immobead 150 variants of *Tl*L or *Rm*L from Sigma-Aldrich], studies with variously immobilized forms of *Ca*LB [13, 17] indicated that hydrophobic adsorption on a modified surface might result in useful biocatalysts. Therefore, the adsorptive immobilization of lipases from *Rhizomucor miehei* and *Thermomyces lanuginosus* (*Rm*L and *Tl*L) was performed as described earlier for *Ca*LA and *Ca*LB [12] as well as for Lipase AK and Lipase PS [17]. Soluble *Rm*L or *Tl*L was diluted in TRIS buffer (pH=7.5) then various grafted silica supports were added to the lipase solution and the resulting suspensions were shaken at 4˚C for 18 h. Activity and selectivity of the immobilized biocatalysts were tested in the enantiomer selective acylation of racemic 1-phenylethanol *rac*-**1** (Fig. 1) and characterized by the specific biocatalyst activity (U_B), conversion (*c*) of the substrate, and enantiomeric excess (*ee*) and enantiomeric ratio (*E*) of the product.

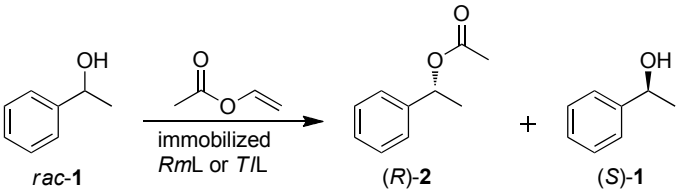

Fig. 1. Kinetic resolution of racemic 1-phenylethanol *rac*-**1** with immobilized *Rm*L or *Tl*L

Conversion (*c*) and enantiomeric excess (*ee*) in the kinetic resolutions of *rac*-**1** were determined by GC. Enantiomeric ratio (*E*) for the reactions was calculated from *c* and $ee_{(R)-2}$[18]. Specific biocatalyst activity (U_B) of the various biocatalysts in the acylation of *rac*-**1** was calculated using the equation $U_B = n_P/(t \times m_B)$ (where n_P [μmol] is the amount of the product (*R*)-**2**, *t* [min] is the reaction time and m_B [g] is the mass of the applied biocatalyst).

3.1 Studies with immobilized *Rhizomucor miehei* lipase (*Rm*L)

First, biocatalysts prepared by hydrophobic adsorption of *Rm*L on mesoporous silica gel, etched silica gel and nineteen variously grafted silica gel supports were investigated (Tab. 1). Because productivity and selectivity differences were most pronounced at low conversions, values after 1 h acylation of racemic 1-phenylethanol *rac*-**1** were used for comparison (Tab. 1).

3.2 Studies with immobilized *Thermomyces lanuginosus* lipase (*Tl*L)

Next, the hydrophobic adsorption of *Tl*L on mesoporous silica gel, etched silica gel and further nineteen variously grafted silica gel supports was studied (Tab. 2). Similarly to the *Rm*L biocatalysts the catalytic properties of our *Tl*L preparations were compared at low conversions. Thus, the bioconversions of racemic 1-phenylethanol *rac*-**1** after 1 h reaction time were evaluated (Tab. 2).

4 4 Discussion

We have found that both *Rm*L (Tab. 1) and *Tl*L (Tab. 2) can be efficiently immobilized on surface-modified mesoporous silica gel supports.

It can be seen that the nature of the silica-gel surface excerted significant influence on the productivity and selectivity of immobilized *Rm*L biocatalyst (Tab. 1). Conversion of the *rac*-**1** to acetate (*R*)-**2** after 1 h reaction time varied between 0.3%–16.3%. Expectedly, when adsorbed onto the untreated or etched

Tab. 1. Kinetic resolution of *rac*-**1** with lipase from *Rhizomucor miehei* (*Rm*L) adsorbed on various surface-modified silica gels (in n-hexane:MTBE 2:1 at 1 h).

Grafting function on silica gel	c [%]	$ee_{(R)-2}$ [%]	E [–]	U_B [μmol min^{-1} g^{-1}]
–	0.4	74.6	6.9	1.2
Etching	0.3	49.3	2.9	0.7
Methyl	6.4	98.4	132	17.6
Ethyl	7.8	98.3	128	21.2
Propyl	16.3	98.5	163	44.9
Isobutyl	0.4	96.6	57	1.0
Hexyl	1.3	96.8	62	3.5
Octyl	2.3	97.9	97	6.3
Decyl	1.8	97.3	76	4.9
Dodecyl	0.8	96.1	59	2.1
Octadecyl	1.8	97.3	74	4.9
Phenyl	7.8	98.6	151	21.5
Perfluorooctyl	10.5	98.5	148	29.0
Vinyl	3.9	98.3	120	10.8
2-Cyanoethyl	3.9	98.6	147	10.7
3-Chloropropyl	6.3	98.9	187	17.2
3-Mercaptopropyl	5.2	98.6	152	14.3
Dimethyl	0.3	98.6	142	0.9
Phenyl-methyl	9.0	98.7	172	24.8
Diphenyl	0.5	68.6	5.4	1.5
Cyclohexyl-methyl	1.6	97.9	96	4.5

Tab. 2. Kinetic resolution of *rac*-**1** with lipase from *Rhizomucor miehei* (*Rm*L) adsorbed on various surface-modified silica gels (in n-hexane:MTBE 2:1 at 1 h).

Grafting function on silica gel	c [%]	$ee_{(R)-2}$ [%]	E [–]	U_B [μmol min^{-1} g^{-1}]
–	1.3	97.9	93	3.6
Etching	1.3	98.1	104	3.6
Methyl	13.5	97.3	84	37.1
Ethyl	17.1	96.9	77	46.9
Propyl	10.6	97.5	90	29.2
Isobutyl	2.3	98.0	99	6.3
Hexyl	6.5	98.0	106	17.8
Octyl	1.2	98.1	105	3.3
Decyl	9.7	97.6	91	26.7
Dodecyl	6.5	98.0	105	18.1
Octadecyl	6.8	97.8	97	18.7
Phenyl	14.0	97.3	85	39.0
Perfluorooctyl	17.8	97.1	82	49.6
Vinyl	14.5	97.0	78	39.7
2-Cyanoethyl	10.4	97.5	90	29.0
3-Chloropropyl	13.9	97.2	81	38.3
3-Mercaptopropyl	14.8	97.1	80	41.1
Dimethyl	13.5	97.2	82	37.2
Phenyl-methyl	20.2	96.6	73	55.9
Diphenyl	4.8	98.0	104	13.2
Cyclohexyl-methyl	10.3	97.8	99	28.7

silica gel, *Rm*Ls showed low productivity (0.4% and 0.3%, respectively). Similarly low values of c were observed with *Rm*Ls on dimethyl-, isobutyl- and diphenyl-grafted supports (0.3%, 0.4% and 0.5%, respectively). The most productive *Rm*L biocatalyst were the ones adorbed on perfluorooctyl- and propyl-grafted silica gels (10.5% and 16.3%, respectively).

Note that the most productive versions of *Rm*L biocatalysts were not the most selective ones (only E = 148 and $ee_{(R)-2}$ = 98.5% for *Rm*L on perfluorooctyl silica and E = 163 and $ee_{(R)-2}$ = 98.5% for *Rm*L on propyl silica). The highest enantiomer selectivity was found for the moderately productive *Rm*L variants i.e. for those on 3-chloropropyl and phenyl-methyl silica (E = 187, $ee_{(R)-2}$ = 98.9% and c = 6.3% and E = 172, $ee_{(R)-2}$ = 98.7% and c = 9.0% respectively).

The catalytic properties of the *Tl*L biocatalysts immobilized on the modified silica-gels depended also significantly on the surface properties of the silica support (Tab. 2). The various *Tl*L biocatalysts catalyzed the conversions of the *rac*-**1** to acetate (*R*)-**2** after 1 h reaction time between 1.2%–20.0%.

Surprisingly, besides the *Tl*Ls on non-treated and etched silica gels (c = 1.3%, for both) *Tl*L on octyl-grafted silica was the least productive (c = 1.3%). The highest productivity was observed when *Tl*L was adsorbed onto phenyl-methyl-, perfluorooctyl- and ethyl-grafted silica gels (20.2%, 17.8% and 17.1%, respectively). Unfortunately, the most active preparations exhibited the lowest enantiomeric selectivity (E = 73, $ee_{(R)-2}$ = 96.6%; E = 82, $ee_{(R)-2}$ = 97.1% and E = 77, $ee_{(R)-2}$ = 96.9%, respectively).

Similarly to the case of *Rm*L adsorption, the highest enantiomer selectivity was found in the acetylation reactions of *rac*-**1** catalyzed by the moderately productive *Tl*L variants adsorbed on longer alkyl chain-modified silica gels (E = 106, $ee_{(R)-2}$ = 98.0% and c = 6.5% for *Tl*L on hexyl silica and E = 105, $ee_{(R)-2}$ = 98.0% and c = 6.5% for *Tl*L on dodecyl silica).

Our results have shown that the optimal method of enzyme immobilization depended both on the nature of the substrate and the reaction conditions [13], [14]]. This study indicated that using a broad selection of variously grafted silica gels for the immobilization of *Rm*L and *Tl*L provided a selection of biocatalysts with a wide range of activity and selectivity when applied for the kinetic resolution of racemic 1-phenylethanol *rac*-**1**.

5 Conclusions

The various mesoporous surface grafted silica gels proved to be efficient supports for the adsorptive immobilization of lipases from *Rhizomucor miehei* (*Rm*L) and *Thermomyces lanuginosus* (*Tl*L) influencing significantly the activity and enantiomer selectivity of the resulting biocatalysts in the kinetic resolution of *rac*-**1**. The fact that in both cases the highest activity or the highest selectivity was achieved with different supports indicated that there is no golden rule for support selection. Although different versions of a particular lipase might be optimal for different substrates, it is expected that the large assortment of the surface modified silica gel supports investigated in this study

can provide us with immobilized *Rm*L and *Tl*L biocatalysts useful in selective biotransformations of other valuable compounds as well.

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